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Role of a major facilitator superfamily transporter in adaptation capacity of *Penicillium funiculosum* under extreme acidic stress

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ABSTRACT

Fungal species present in extreme low pH environments are expected to have adapted for tolerance to high H^+ concentrations. However, their adaptability mechanism is unclear. In this study, we isolated an acid-tolerant strain of *Penicillium funiculosum*, which can grow actively at pH 1.0 and thrived in pH 0.6. A major facilitator superfamily transporter (PfMFS) was isolated from an acid-sensitive random insertional mutant (M4) of the fungus. It encodes a putative protein of 551 residues and contains 14 transmembrane-spanning segments. A targeted mutant (M7) carrying an inactivated copy of *PfMFS* showed an obvious reduction of growth compared with the wild type (WT) and complementation of M7 with *PfMFS* restored the wild-type level of growth at pH 1.0. Further data showed that the wild-type showed higher intracellular pH than M7 in response to pH 1. Subcellular localization showed that PfMFS was a cell membrane protein. Homology modeling showed structural similarity with an MFS transporter EmrD from *Escherichia coli*. These results demonstrate that the PfMFS transporter is involved in the acid resistance and intracellular pH homeostasis of *P. funiculosum*.

1. Introduction

In nature, living organisms can be found over a wide range of extreme environments, such as high temperature, acidity/alkalinity, salinity, pressure and radiation. The organisms that thrive in extreme environments are extremophiles. They are found in all three domains of life (Archaea, Eubacteria and Eukaryotes) (Rothschild and Mancinelli, 2001; Magan, 2007). Most of extremophiles belong to the domain Archaea and the domain Bacteria, specifically to the domain Archaea. Extremophiles from the Eukarya domain can also be found living in extreme environments. Most eukaryotic extremophiles belong to the fungal and algal classes. Extremophiles play important roles in many ecosystems and provide rich microbial resources for human exploitation. The study of extremophiles has provided ground-breaking discoveries that challenge the paradigms of modern biology and make us rethink intriguing questions. The mechanisms by which different microorganisms adapt to extreme environments provide a unique perspective on the fundamental characteristics of biological processes present in most species. Extremophiles are also critical for evolutionary studies related to the origins of life. Furthermore, the application of extremophiles in industrial processes has opened a new era in biotechnology.

Acidophiles or acidophilic organisms are a kind of extremophiles which thrive under highly acidic conditions (usually at pH 2.0 or below). A strict definition is that acidophiles are organisms which are able to grow down to pH 1.0 and are able to actively grow at pH < 4.0 (Magan, 2007). Especially, acidophilic archaea and acidophilic bacteria are defined as organisms that have a pH optimum for growth of less than pH 3 (Baker-Austin and Dopson, 2007). These organisms can be found in all domain of life, including Archaea, Bacteria, and Eukaryotes. Among eukaryotic organisms, only a few species of fungi have so far been known to grow below pH 1, such as *Acontium cylatium*, *Cephalosporium* species and *Trichosporon cerebriae* (Rothschild and Mancinelli, 2001).

Organisms that live at extremely low pH are able to maintain their cytoplasm at neutral or near neutral pHs (Baker-Austin and Dopson, 2007; Bignell, 2012; Hesse et al., 2002). This intracellular pH homeostasis is achieved by a variety of mechanisms that involve restricting proton entry by the cytoplasmic membrane and purging of protons and their effects by the cytoplasm. Major categories of active pH homeostasis mechanisms include: a reversed membrane potential, highly impermeable cell membranes and proton efflux systems containing primary active transporters (such as proton ATPases) and secondary transporters (Rothschild and Mancinelli, 2001; Magan, 2007; Baker-Austin

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and Dopson, 2007; Bignell, 2012; Kanjee and Houry, 2013; Brett et al., 2011; Krulwich et al., 2011). In fungi, an ATP-dependent proton ATPase, Pam1, actively pumps protons out of the cell. Another ATP-dependent proton ATPase is the vacuolar ATPase (V-ATPase) which drives transport of protons into the vacuole (Bignell, 2012).

Among intracellular pH homeostasis mechanisms, secondary transporters have received attention because of their predominance in acidophile genome sequences (Futterer et al., 2004; Tyson et al., 2004; Crossman et al., 2004). They are believed to be an important functional adaptation of organisms to survive in an extremely acidic environment. Secondary transporters are membrane proteins that use the transmembrane electrochemical gradient of protons or sodium ions to drive transport rather than by ATP hydrolysis. Since the driving force (proton or sodium gradient) is normally directed inwards, antiport mechanisms are generally used to export substrates, while symport mechanisms operate for import. The class of secondary transporters contains many different families, but most members belong to the major facilitator superfamily (MFS) and the amino acid–polyamine–organocation (APC) superfamily. The major facilitator superfamily is the first largest superfamily of secondary transporters and includes members that function as uniporters, symporters or antiporters. The MFS superfamily consists of 74 families, each of which is usually concerned with the transport of a certain type of substrate (simple monosaccharides, oligosaccharides, amino acids, peptides, vitamins, enzyme cofactors, drugs, chromophores, nucleobases, nucleosides, nucleotides, iron chelates, and organic and inorganic anions and cations) (Reddy et al., 2012). Most MFS proteins vary between 400 and 600 amino acid residues in length and possess either 12 or 14 putative transmembrane segments (TMS) (Law et al., 2008). It has been demonstrated that some MFS proteins from fungi play a key role in resistance to natural toxic compounds and fungicides (Alexander et al., 1999; Prasad and Kapoor, 2005; Roohparvar et al., 2007; Hayashi et al., 2002; Coleman and Mylonakis, 2009; Liu et al., 2012) and virulence (Pitkin et al., 1996; Callahan et al., 1999; Coleman and Mylonakis, 2009). However, whether the MFS proteins are involved in acid resistance or intracellular pH homeostasis is unclear.

The APC superfamily appears to be the second largest superfamily of secondary transporters. The APC superfamily has been shown to include five recognized families, four of which are specific for amino acids and their derivatives (Wong et al., 2012). Currently, it has been well demonstrated that two secondary transporters of the APC superfamily, Adic and GadC, which are major components of the acid-resistance system 2 (AR2) and the acid-resistance system 3 (AR3) in *Escherichia coli*, are involved in acid resistance of *E. coli* (Richard and Foster, 2004; Foster, 2004; Ma et al., 2012; Gao et al., 2009, 2010; Kanjee and Houry, 2013). Apart from the two secondary transporters Adic and GadC, Na^+/H^+ antiporters belonging to secondary transporters from the NHE superfamily are also involved in intracellular pH homeostasis. Over-expression or deletion of a Na^+/H^+ antiporter Nhx1 in *Saccharomyces cerevisiae* resulted in alkalinization or acidification of vacuole pH, respectively (Brett et al., 2005; Ali et al., 2004). A Na^+/H^+ antiporter NhaA is essential for intracellular pH regulation in *E. coli* under alkaline conditions (Padan et al., 2005; Hunte et al., 2005; Mager et al., 2011).

Fungal species present in extreme low pH environments are expected to have adapted for tolerance to high H^+ concentrations. However, their adaptability mechanism is unclear. One important scientific question is how they adapt such an extreme low pH environment, by the above mechanisms or others? In this study, we isolated an acid-tolerant fungal strain of *Penicillium funiculosum* X33, which can grow actively at pH 1.0 and thrived in pH 0.6. We investigated the involvement of a major facilitator superfamily transporter (PfMFS) in adaptation for tolerance to high H^+ concentrations by *P. funiculosum*. Using insertional gene mutagenesis, targeted gene disruption and complementation of disrupted mutants along with cellular pH analysis, we demonstrate that the major facilitator superfamily transporter is involved in the acid resistance and intracellular pH homeostasis of *P. funiculosum*.

2. Material and methods

2.1. Strain, media and growth conditions

The fungus *P. funiculosum* X33 was originally isolated from an acidic environment in China, China vinegar with an extremely low pH 2.2. The fungus was inoculated to potato-dextrose agar (PDA: 10 g glucose, 20 g potato and 15 agar in 1 L water) plates at 28 °C and different pH or potato-dextrose broth (PDB: 10 g glucose and 20 g potato in 1 L water) medium at 28 °C and pH 1 under static culture conditions. The pH of the PDA plates and PD media was adjusted to 0.0–14.0 with H_2SO_4 and NaOH. The different pH PDA plates and PDB media were inoculated with the fungus from freshly grown PDA plates. *E. coli* DH5α was used for DNA manipulations and transformations. *Agrobacterium tumefaciens* strain LBA4404 and EHA105 were used as a T-DNA donor for fungal transformation and gene location, respectively.

To quantificationally estimate growth of *P. funiculosum* X33 at low pH, the following experiments were carried out. Growth of *P. funiculosum* were performed by inoculating 10 ml of PDB medium (pH 1.0) in a 50 ml flask with spore suspension. Growth was monitored by measuring fresh weight after culturing for 5 d at 28 °C.

2.2. DNA manipulation and sequence analysis of gene PfMFS

Standard molecular techniques were performed according to Molecular Cloning (Sambrook and Russell, 2001). Fungal DNA and RNA were extracted by the CTAB protocol or Trizol protocol, respectively. Bacterial plasmid DNA was isolated using the plasmid mini kit (OMIGA), according to the manufacturer's suggestion. Primers were designed using Primer Premier 5.0 software. The molecular mass of the mature peptide was calculated using Vector NTI 7.0 software.

2.3. Fungal isolation and growth on PDA with different pH value

The fungal strain *P. funiculosum* X33 was isolated from extreme acid conditions in our laboratory. The medium for isolation was PDA. The taxon of strain *P. funiculosum* was identified based on morphology and on rDNA sequence in the internal transcribed spacer (ITS) regions. To test the growth pH values range of the *P. funiculosum*, the strain was inoculated on PDA plates and PDB with pH values ranging from 0.5 to 13.0. The pH of the medium was regulated with H_2SO_4 and NaOH.

2.4. ATMT transformation and screening for mutants and cloning of the gene

A. tumefaciens strain LBA4404 harboring the pROK2/*hph* was used for transforming *P. funiculosum* as previously described (Han et al., 2012). The putative transformants were transferred to PDA plates containing 1000 μg/ml hygromycin B and 300 μg/ml SM. The insert of T-DNA was conformed by PCR analysis using a pair of primers (hph1/hph2) and Southern bolting with probe *hph*. The conformed transformants were then transferred to PDB media of pH 1.0, the mutants which poorly grow in pH 1.0 media were appeared after 7 days incubation in 28 °C.

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed for the cloning of T-DNA flanking sequences from *P. funiculosum* mutants. The specific right border (RB) primers (TR1, TR2, and TR3) and left border (LB) primers (TL1, TL2, and TL3) and five arbitrary degenerate primers (AD1, AD2, AD3, AD4 and AD5) used (Supplementary Table 1) were designed as described previously (Liu et al., 1995). The reaction conditions and thermal cycling settings followed the protocol of Liu and Huang (1998). The tertiary TAIL-PCR product of each transformant showing the highest intensity was purified using Qiagen Gel Extraction kit (Qiagen) and sequenced at Shenggong (Shanghai, China). Sequence comparison was performed using the BLAST programs available on the NCBI web site (http://www.ncbi.nlm.nih.gov).

2.5. PfMFS disruption and reverse complementation

For construction of the disrupt vector pROK2/PfMFS, a 1.2-kb *Sph*I/*Hind*III fragment containing the 3′ flanking sequences of the PfMFS was obtained by PCR-amplification, using genomic DNA of *P. funiculosum* as template and a pair of primers (MFSX-F and MFSX-R). After digestion with the appropriate restriction enzymes, the fragment was inserted into *Sph*I/*Hind*III-cut pUCATPH (Lu et al., 1994). Subsequently, the resulting plasmid was digested with *Kpn*I/*Sal*I and a 1.2-kb fragment containing the 5' flanking sequences of the PfMFS gene was obtained by PCR amplification with a pair of primers (MFSS-F and MFSS-R) and inserted. The whole disruption cassette containing the hygromycin B phosphotransferase (*hph*) gene was then excised from the resulting plasmid by digestion with *Hind*III/*Kpn*I, and the cassette was ligated with *Hind*III/*Kpn*I-cut pROK2 resulting in the binary disruption vector pROK2/*PfMFS*. Finally, the constructed disruption vector was transformed into *A. tumefaciens* LBA4400 by electroporation (Mozo and Hooykaas, 1991) and transformants were selected on LB agar with spectinomycin (250 μg/ml) and kanamycin (100 μg/ml). *Agrobacterium* strains containing the binary vector was identified by PCR, applying a pair of primers (MFSS-F and MFSX-R) for identification of pROK2/*PfMFS*. The resultant final plasmid construct was mobilized into *A. tumefaciens* LBA4404 for fungal transformation. PfMFS disruption mutants were initially selected on the basis of hygromycin B resistance. PCR performed with a pair of primer pairs (HR1/HR2 and HB1/HB2) (Supplementary Table 1), Southern blotting and RT-PCR were used to confirm disruption of PfMFS in *P. funiculosum* transformants.

To complement the PfMFS mutant, the *PfMFS* gene containing its upstream and downstream sequences was amplified with a pair of primers (HB1 and HB2) (Supplementary Table 1). After being confirmed by sequencing, the resultant PCR product was digested with *Sal*I and inserted into *Sal*I sites of p3SR2 to form p3SR2/*PfMFS*. This plasmid was used for fungal transformation (Beri and Turner, 1987). Reverse complementation transformants were initially selected on acetamide medium. PCR performed with a pair of primers (HB1 and HB2) (Supplementary Table 1) was then used to confirm the insertion of the wild-type PfMFS. The expression of PfMFS was then confirmed by RT-PCR with a pair of primers (RH1 and RH2) (Supplementary Table 1) and Northern blot analysis.

2.6. DNA extraction and Southern blot analysis

Fungal genomic DNA was extracted from the mycelia following the method described with DIG High Prime labeling Kit according to the instructions of manufacturer (Roche Applied Science). To verify the integration of T-DNA in fungal transformants, transformants were analyzed by PCR using the *hph*-specific primers. Transformants were subjected to Southern blot analysis to dissect the integration types of targeting cassettes. The genomic DNA (2–5 μg) was cut by different restriction enzymes. The digested DNA fragments were separated on a 0.8% (w/v) agar gel and blotted onto Hybond N^+ membrane (Amersham Pharmacia Biotech, Ireland) by capillary action according to the method (Sambrook and Russell, 2001). Labeling of probe, hybridization, and color detection were performed with DIG High Prime labeling Kit II according to the instructions of manufacturer (Roche Applied Science).

2.7. RNA extraction, RT-PCR analysis and Northern blot analysis

The total RNA of the strains WT and mutants were extracted from the 5-day-old mycelia grown on PDB by the Trizol protocol (Life Technologies). Two-step RT-PCR was performed using a RT-PCR Kit (Takata). Northern blot analysis was performed according to the instructions of manufacturer DIG Northern Starter Kit (Roche Applied Science).

2.8. Subcellular localization of PfMFS

For observation of the subcellular localization of PfMFS in living cells, the full-length ORF of PfMFS cDNA amplified with a pair of specific primers (DW1 and DW2) containing restriction sites (Supplementary Table 1) was fused to the upstream of in the pROK2/35S-*GFP* plasmid. The resulting recombinant plasmids were designated as the pROK2/35S-*PfMFS-GFP* plasmid. The constructed plasmids were transformed into *A. tumefaciens* LBA4400 by electroporation. *A. tumefaciens* LBA4400 cells carrying pROK2/35S-*GFP* or pROK2/35S-*PfMFS-GFP* were used for expression of GFP-tagged PfMFS in onion epidermal cells via *A. tumefaciens* infiltration. A 50-ml overnight culture of *A. tumefaciens* was collected by centrifugation and resuspended in 20 ml of 0.5X MS medium supplemented with 150 mM acetosyringone. After 3 h growth at 28 °C. Onion bulb slices were surface-sterilized in 75% (v/v) ethanol for 1 min and washed with 1X MS liquid medium, followed by treatment with 2% (w/v) sodium hypochlorite for 2 min. After four times of washing with 1X MS liquid medium, the onion slices were treated with 4 M NaCl for 20 min, and then incubated with the *A. tumefaciens* cell culture for 90 min. After removal of the surface liquid, onion slices were placed onto MS medium-agar plates and grown at 26 °C for 48 h in the dark. Fluorescence of GFP was observed by a fluorescent microscope.

To further confirm the subcellular localization of PfMFS in *P. funiculosum* living cells, the full-length ORF of PfMFS cDNA amplified with a pair of specific primers (FDW1 and FDW2) containing restriction sites (Supplementary Table 1) was fused to the upstream of in the pSur-trpC-*GFP* plasmid. The resulting recombinant plasmids were designated as the pSur-trpC-*PfMFS-GFP* plasmid. The constructed plasmids were transformed into *A. tumefaciens* LBA4400 by electroporation. *A. tumefaciens* LBA4400 cells carrying pSur-trpC-*GFP* or pSur-trpC-*PfMFS-GFP* transformed *P. funiculosum* cells for expression of GFP-tagged PfMFS in *P. funiculosum* cells. Fluorescence of GFP was observed by a fluorescent microscope.

2.9. Measurement of cellular pH and cell suspension pH

Cultures of *P. funiculosum* wild type (WT) and *PfMFS* disruption mutant (M7) were grown 3 d at 28 °C in PDB medium at 7.0. Culture was centrifuged at 4000 rpm. The cultured mycelia were washed once with 0.7 NaCl (pH 7.0). Then 0.7 NaCl (pH 7.0) containing lysozyme (1 mg/ml) (Sigma) was added. To form protoplasts, the cells were incubated at room temperature in a shaker at low speed. After 4 h the incubated cells were separated by centrifugation at 2000 rpm for 20 min. The protoplasts were resuspended in 0.7 M NaCl (pH 7.0) containing 50 μM BCECF-CM, and incubated at 28 °C for 30 min. The protoplasts were collected again by centrifugation, washed three times with 0.7 M NaCl (pH 7.0),

resuspended in 200 μl of 0.7 M NaCl (pH 1.0) and incubated for 45 min at 28 °C, and then used for fluorescence measurement. Fluorescence intensity values were measured using methods previously described (Ali et al., 2004). The absolute values of cellular pH shown were estimated after calibration (Ali et al., 2004).

2.10. Nucleotide sequence accession numbers

The nucleotide sequence for the *P. funiculosum* ITS regions and the *PfMFS* gene were deposited in the GenBank database under accession numbers GQ422445 and KF815490, respectively.

3. Results

3.1. Identification of the fungal strain isolated and assay of its growth pH range

The fungus belongs to a species of the genus *Penicillium*. Colonies are deep-green. Reverse is red or purple to almost black. Conidiophores arise laterally form aerial hyphae and are smooth-walled. The phialides are verticillate in dense groups and cylindrical. Conidia are elliptical, green and smooth. These morphological characteristics are similar to those of *P. funiculosum* (Miller et al., 1957). The PCR-amplified rDNA ITS sequence (GQ422445) of the fungus revealed the highest nucleotide identity (99%) with that from *P. funiculosum* strain TS08 (GU980968), *P. funiculosum* strain C2-20 (JQ717348) and *P. funiculosum* strain C2-19 (JQ717333). Based on morphology and ITS sequence analysis, the strain was identified as *P. funiculosum* X33.

In order to obtain information regarding the influence of extreme acidity and alkalinity on growth, the fungus was grown in different pH PDA plates and PDB media. Our result showed that the fungus grew well from pH 2.0 to 12.0, and could grow at pH 0.6, 0.7, 0.8, 0.9, 1.0 and 13.0, and no growth was observed at and below pH 0.5 and at pH14 (Fig. 1). The result indicates that the fungus has the widest range of pH values from pH 0.6 to pH 13 for growth in life. To our knowledge, this is the first report on fungi growing not only at pH 0.6 but also at pH 13.

3.2. Insertional mutagenesis and mutant selection

The fungal *P. funiculosum* was able to grow actively at pH 1.0. To obtain mutants of growth reduction compared with the wild type at pH 1.0, *A. tumefaciens* was used to transform the fungus by random integration of transforming DNA (T-DNA). More than 19,300 stable transformants were obtained on PDA containing 1000 μg/ml hygromycin B. The transformants were then cultivated on the PDB with pH value of 1.0. Through the section of these transformants, we found that a transformant M4 showed an obvious reduction of growth compared with the wild type (Fig. 2A). PCR analysis of the transformant M4 demonstrated that the hygromycin resistance cassette was integrated in the genome of *P. funiculosum* (Fig. 2B). Southern blot analysis revealed that the transformant M4 was single T-DNA integrated randomly in the genome (Fig. 2C).

3.3. Isolation of the gene encoding a MFS family transporter from P. funiculosum and sequence analysis

Isolation of unknown DNA sequences flanked by known sequences is an important task in molecular biology research. Thermal asymmetric interlaced PCR (TAIL-PCR) is an effective method for this purpose (Mullins et al., 2001). In this study, the mutant M4 was used for isolation of disrupted gene. Using TAIL-PCR, we obtained left and right border flanking sequences in the insertion site in M4. The PCR products of the left and the right border sequences were 906 bp and 331 bp, respectively (Supplementary Fig. 1A and B). To determine the precise junction of the left and the right border sequences, we amplified the left and the right border sequences using a pair of specific primers from the fungus wild strain genomic DNA, and a fragment with 789 bp in size was obtained and sequenced (Supplementary Fig. 1C). The fragment sequence contained both the left and the right border sequences and was identical with the spliced left and the right border sequences. Through subsequent rounds of TAIL-PCR using nested insertion-specific primers, we obtained a 3789-bp final fragment sequence (Supplementary Fig. 1D). The 3789-bp sequence showed 88% identity to *Penicillium marneffei* ATCC 18224 conserved hypothetical protein (XP_002144911). It contained an open reading frame of 1656 bp encoding a putative 551-amino-acid protein. Three introns of 61 bp, 72 bp and 61 bp were identified based on alignment with the homologous conserved hypothetical protein (XP_002144911), and their position was confirmed by RT-PCR. Sequence analysis showed that the 5′-untranslated region (UTR) of the MFS transporter contained three putative TATA elements at −72, −89 and −600 and a putative CCAAT box at position −1068. The calculated molecular weight and pI of the encoded putative mature protein were 59.5 kDa and 6.08, respectively. BLASTP analysis showed the

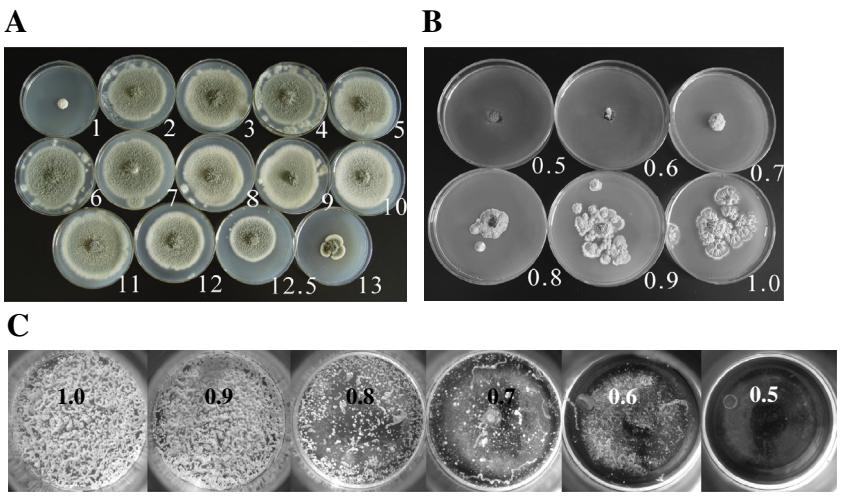

Fig. 1. Growth of *Penicillium funiculosum*. (A) *P. funiculosum* cultivated on PDA for 7 d with different pH values range form 1.0 to 13.0. (B), *P. funiculosum* cultivated on PDA for 20 d with different pH values range form 0.5 to 1.0. (C), *P. funiculosum* cultivated on PDB for 7 d with pH values range form 0.5 to 1.0 in 28 °C.

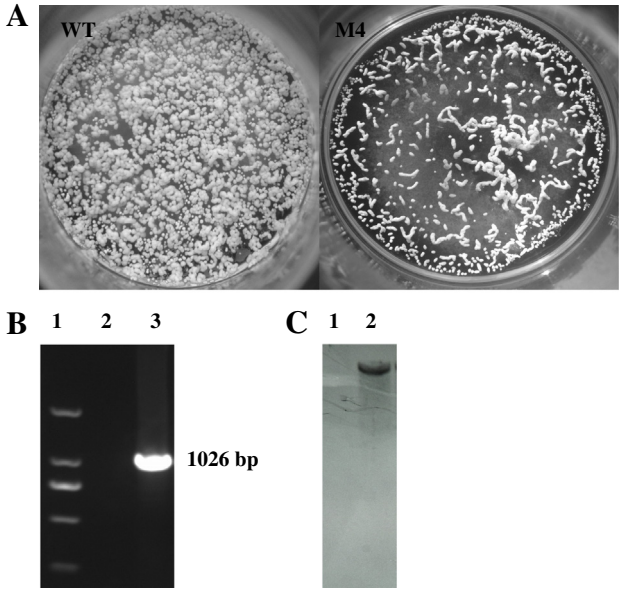

Fig. 2. (A) Colony growth of *Penicillium funiculosum* wild-type strain (WT) and transformant strain (M4) obtained through insertional gene mutagenesis on PDB for 7 d at pH 1.0. (B) PCR amplification for *hph* gene with a primer of pairs hph1/hph2 from the genome DNA of the strains. Lane 1, DNA ladder; Lane 2, WT; Lane 3, M4. (C) Southern blot analysis of the *hph* gene. Genomic DNA was digested with HindIII. Lane 1, WT; Lane 2, M4.

encoded putative protein belongs to the major facilitator superfamily (MFS). The putative protein exhibited high homology to MFS transporters and shared 82%, 70%, 69% and 65% identity with *P. marneffei* ATCC 18224 conserved hypothetical protein (XP_002144911), *Aspergillus oryzae* putative transporter (EIT74145), *A. oryzae* RIB40 unnamed protein product (BAE55697) and *Aspergillus niger* CBS MFS transporter (XP_001398241), respectively. Alignment of the MFS transporter and other MFS transporters from fungal species indicated that conserved motifs were found in the sequence of the MFS transporter (Supplementary Fig. 2). Phylogenetic analysis indicated that the MFS transporter had the closest relationship with conserved hypothetical protein (XP_002144911) (Supplementary Fig. 3). The prediction results with ProtFun 2.2 Server showed that the putative protein was a transporter. TMMTOP prediction result showed that the MFS transporter had 14 predicted transmembrane-spanning segments connected by hydrophilic loops, with both their N- and C-termini located in the cytoplasm (Supplementary Fig. 4), suggesting that it was a transmembrane protein. These search results indicate that the putative protein is a novel MFS transporter, but its functional significance is unclear. We named the novel MFS transporter as PfMFS.

3.4. PfMFS was involved in P. funiculosum to grow in the low pH value

In this study, we obtained the mutant M4, which showed obviously reduced growth compared with the wild type at pH 1.0, using T-DNA random insertional mutagenesis. We identified and characterized its corresponding mutated gene, *PfMFS*. Sequence analysis of the genomic DNA franking T-DNA isolated by TAIL-PCR technique showed that the T-DNA insert site in the mutant M4 was located in the promoter region of *PfMFS* gene at position −372. The result indicates that *PfMFS* gene is involved in acid resistance of *P. funiculosum*.

To further confirm the function of the *PfMFS* in *P. funiculosum* acid-tolerance, *PfMFS* was disrupted by homologous recombination. The *PfMFS* gene replacement vector pROK2/*PfMFS* was constructed by replacing a 500-bp sequence in the *PfMFS* coding region with the hygromycin cassette from the plasmid pUCATPH. The hygromycin-resistant transformants were transferred to PDA plates containing 1000 μg/ml hygromycin B, and were subjected to PCR and Southern blot analysis. Among these transformants, we selected a transformant M7 showing an obvious reduction of growth compared with the wild type (Fig. 3). In the gene replacement experiments, the mutant M7 was identified harboring a *PfMFS* disruption as determined by PCR (Fig. 4A and B). From Southern blot analysis of the genomic DNA digested with different restriction enzymes and probed with *PfMFS*, *PfMFS* was a single-copy gene in the *P. funiculosum* genome (Fig. 4C). The *PfMFS* gene disruption was validated by Southern blot analysis (Fig. 4C). RT-PCR and Northern analysis of the mutant confirmed that *PfMFS* expression was inhibited (Fig. 4D and E). On PDB with pH value of 1.0, *PfMFS* disruption was distinguishable from wild-type *P. funiculosum* in terms of growth (Fig. 3). These data further demonstrate that *PfMFS* is required for *P. funiculosum* acid-tolerance.

3.5. Complementation of PfMFS

Though two independent mutants (M4 and M7) showed the same phenotype, we decided to perform a complementation test of *PfMFS* to be sure that whether the mutant phenotype could be complemented by introduction of the wild-type *PfMFS* gene. We transformed the disrupted mutant M7 with the complementation vector p3SR2/*PfMFS*. Complemented transformants were selected on acetamide media. A complemented transformant M7-5 was confirmed by PCR with a pair of primers specific for *PfMFS*. The expression of *PfMFS* was confirmed by RT-PCR and Northern blot analysis (Fig. 4D and E). It was obvious that the complemented transformant M7-5 contained *PfMFS*, like the wild-type strain. This complemented strain grew at the wild-type growth rate on PDB with pH value of 1.0 (Fig. 3). The data indicate that disrupted *PfMFS* is responsible for the phenotype observed in the two mutants, M4 and M7.

3.6. Cellular pH analysis with the PfMFS disruption mutant

To examine PfMFS function for cellular pH homeostasis, cellular pH was determined in the wild type and the PfMFS disruption mutant M7 of *P. funiculosum* at pH 1.0 using highly fluorescent BCECF that is particularly sensitive to pH. The wild-type strain was found to show higher intracellular pH than the PfMFS disruption mutant M7 in response to pH 1 (Fig. 5). Growth sensitivity to pH 1.0 in the PfMFS disruption mutant M7 could be due to defective pH homeostasis. These results show that PfMFS is important for cellular pH homeostasis.

3.7. PfMFS is targeted to the cell membrane

The deduced PfMFS protein contains no nuclear localization signals (NLS) as predicted by PSORT analysis (Nakai and Kanehisa, 1992). To examine cellular distribution of PfMFS, we fused PfMFS with green fluorescent protein (GFP) and the chimeric gene was put under the control of CaMV35S promoter. The resulting plasmid pROK2/PfMFS-GFP was then transformed into onion epidermal cells. As shown in Fig. 6A and B, localization of the PfMFS-GFP fusion protein was visualized exclusively in the cell membrane, whereas the control GFP (pROK2/GFP) was distributed both in the cytoplasm and the nucleus. To further confirm the subcellular localization of PfMFS in *P. funiculosum* cells, *P. funiculosum* cells were transformed by *A. tumefaciens* LBA4400 cells carrying pSur-trpC-*PfMFS-GFP*. Localization of the PfMFS-GFP fusion protein was also visualized in the cell membrane in *P. funiculosum* cells, whereas the control GFP (pSur-trpC-*GFP*) was distributed in the cytoplasm in *P. funiculosum* cells (Fig. 6C and D). These results indicate that the PfMFS transporter is a cell membrane protein.

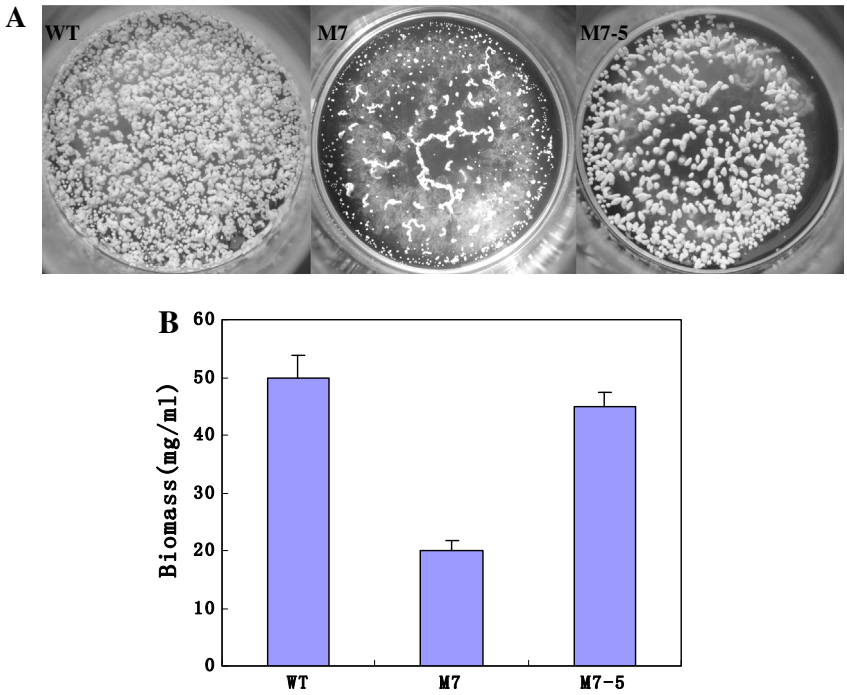

Fig. 3. Colony morphology and biomass of the wild-type strain (WT), the *PfMFS* gene disruption mutant strain (M7) and the complemented strain (M7-5) of *Penicillium funiculosum* growing on PDB with pH 1.0. The photographs were taken and the biomass was measured after 5 days of incubation at 28 °C. (A) Colony morphology and (B) biomass (mg fresh weight/ml).

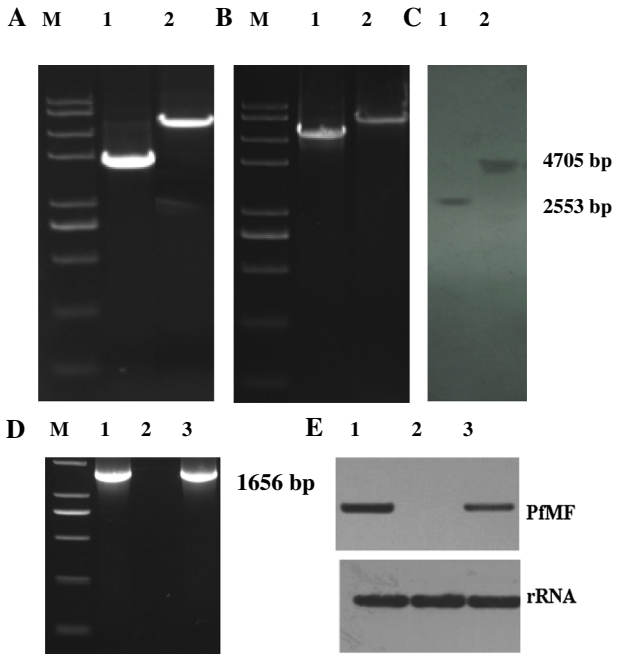

Fig. 4. Confirmation of the mutant targeted disruption and expression analysis of the *PfMFS* gene and expression analysis of the *PfMFS* gene at pH 1.0. (A) PCR amplification for *PfMFS* gene with a primer of pairs HR1/HR2 from the genome DNA of strains. Lane 1, the wild-type strain; Lane 2, the *PfMFS* mutant M7. The wild-type obtained 1850 bp, the *PfMFS* mutant M7 obtained 3490 bp. (B) PCR amplification for *PfMFS* gene with a primer of pairs HB1/HB2 from the genome DNA of strains. Lane 1, the wild-type strain; Lane 2, the *PfMFS* disruption mutant (M7). The wild-type obtained 3450 bp, the *PfMFS* mutant M7 obtained 5080 bp. (C) Southern blot analysis of the *PfMFS* gene. Genomic DNA was digested with *Sph*I and *Eco*RV. Lane 1, the wild-type strain; Lane 2, the *PfMFS* disruption mutant M7. (D) RT-PCR products of the *PfMFS* gene in total RNA with a primer of pairs HR1/HR2. Lane 1, the wild-type strain; Lane 2, M7; Lane 3, the complemented strain (M7-5). (E) Northern blot analysis of the *PfMFS* gene in total RNA. Lane 1, the wild-type strain; Lane 2, M7; Lane 3, M7-5.

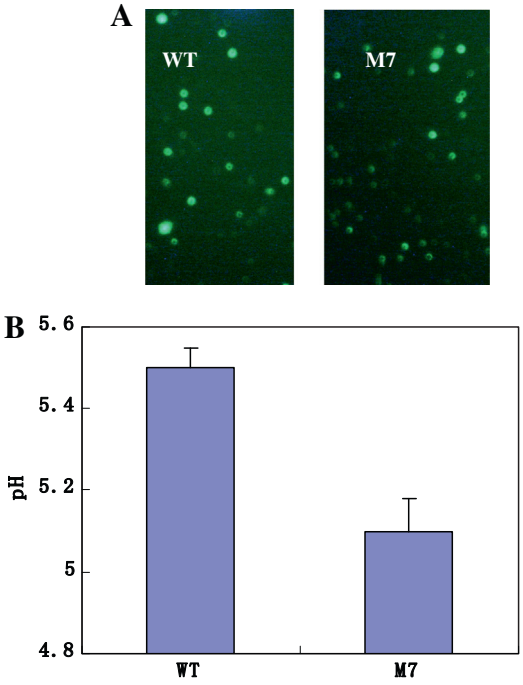

Fig. 5. Cellular pH analysis of protoplasts from *Penicillium funiculosum* wild type (WT) and *PfMFS* disruption mutant (M7) treated with pH 1.0. (A) *P. funiculosum* protoplasts were visualized under a fluorescence microscope with fluorescence. (B) Cell pH values were estimated using methods previously described (Ali et al., 2004).

3.8. Homology modeling of PfMFS by Swiss-Model

So far, crystal structure of only three MFS members, EmrD, LacY and GlpT, was solved from bacteria (Baker et al., 2012). Unfortunately, no crystal structure of the major facilitator superfamily transporters is determined from fungi. In this study, sequence alignment showed that the PfMFS transporter shared the highest

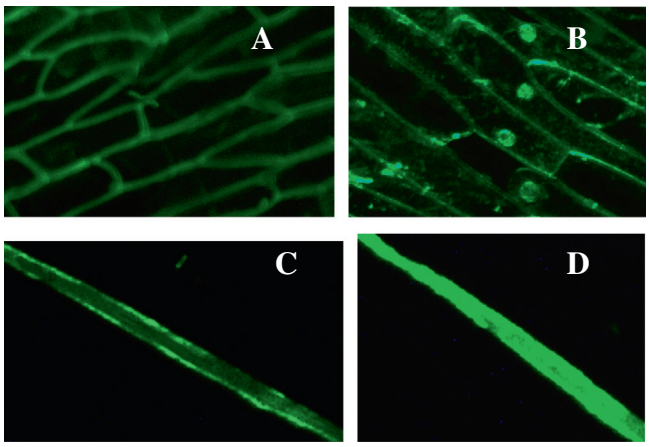

Fig. 6. Subcellular localization of the PfMFS from *Penicillium funiculosum*. (A) Transient expression of PfMFS-GFP fusion protein in the epidermis cells of onion. The fusion protein is present in the cell membrance. (B) Transient expression of GFP protein in the epidermis cells of onion. The GFP protein is present in the cell outlines and the nuclear. The epidermis cells of onion were visualized under a fluorescence microscope with fluorescence. (C) Expression of PfMFS-GFP fusion protein in *Penicillium funiculosum* cells. The fusion protein is present in the cell membrance. (D) Expression of GFP protein in *Penicillium funiculosum* cells. The GFP protein is present in the cell outlines. *Penicillium funiculosum* mycelium cells were visualized under a fluorescence microscope with fluorescence.

identity (16.09%) and similarity (27%) with EmrD among the three members of the MFS (Fig. 7A). The degree of identity and similarity of amino acid sequence allowed the generation and the prediction of a plausible 3-D model for the PfMFS transporter by Swiss-Model (an automated comparative protein modeling server) using EmrD as a template (Yin et al., 2006). The homology model shows that the over fold of the PfMFS transporter may be a large central cavity (Fig. 7B and C), and similar to the over fold of the template EmrD (Yin et al., 2006).

4. Discussion

P. funiculosum has been isolated from various substrates and habitats. Its growth can occur from 8 to 42 °C with an optimum at 25–28 °C. Acid-tolerant species is able to develop at pH 3.0–3.7 (Gross and Robbins, 2000). *P. funiculosum* strain X33 was isolated from a kind of vinegar (pH 2.4) in China. Historically, vinegar originates from China. About 3000 years ago, it was recorded by 'the Rites of Zhou', which is an important magnum opus in ancient China. Therefore, vinegar has history of at least 3000 years. In the long time, the fungus has possibly adapted low-pH habitats through mutation and selection, and could survive by producing a number of spores. As to growth of the fungus at extremely high pH, the fungus might possibly face a high alkali environment in its evolution, or evolve from an alkalophilic member of *Penicillium*. Interestingly, what mechanisms cause the fungus to survive under such extremely and widely pH environments.

Sequencing and annotation of the genome of the extremely thermoacidophilic archaebacterium *Picrophilus torridus*, able to grow at around pH 0, has revealed further insights into life in such extreme acidic conditions (Futterer et al., 2004). About more than 10% of the genome encodes secondary transporters (Futterer et al., 2004). The numerous secondary transporters probably represent a strategy for the adaptation of these organisms to extremely acidic environment (Futterer et al., 2004; Crossman et al., 2004). It has been well demonstrated that two secondary transporters of the amino acid–polyamine–organocation (APC) superfamily, Adic and GadC, were involved in acid resistance of *E. coli* (Richard and Foster, 2004; Foster, 2004; Ma et al., 2012; Gao et al., 2009, 2010). In this study, we described the cloning and functional characterization of PfMFS, the first major facilitator superfamily (MFS) transporter gene cloned from *P. funiculosum*. We demonstrated that PfMFS was involved in acid resistance and intracellular pH homeostasis of *P. funiculosum*. This is the first report about the involvement of major facilitator superfamily transporters in acid resistance and intracellular pH homeostasis.

It is interesting that how the PfMFS transporter can contribute to acid resistance and intracellular pH homeostasis of *P. funiculosum*. It has been demonstrated that the mechanism of acid resistance in *E. coli* is implemented through the decarboxylase/antiporter-dependent acid-resistance systems (AR2 and AR3) (Richard and Foster, 2004; Foster, 2004). The glutamate decarboxylase isozymes GadA and GadB or the arginine decarboxylase AdiA replace the alpha-carboxyl groups of their amino acid substrates (glutamate and arginine) with a proton from the cytoplasm. CO_2 and gamma-amino butyric acid (GABA) or agmatine are produced. The cognate antiporter GadC for glutamate and the cognate antiporter AdiC for arginine expel the decarboxylation products in exchange for importing new amino acid substrates. The removal of intracellular protons by this mechanism caused internal pH to increase to levels that would not damage critical cell components. Recent structural and biochemical analysis of AdiC and GadC also supports the acid-dependent acid resistance systems (Gao et al., 2009; Ma et al., 2012). In contrast to the acid resistance and pH homeostasis mechanism of the two secondary transporters Adic and GadC in *E. coli*, that of two secondary transporters Na^+/H^+ antiporters Nhx1 in *S. cerevisiae* and NhaA in *E. coli* is not involved in decarboxylase and decarboxylation. Nhx1 and NhaA transporters belong to the NHE superfamily. Their main physiological role is the regulation of cytoplasmic pH and Na^+ content for growth and development througt a direct ion-exchange machinery bewteen Na^+ and H^+, drived by proton motive force (PMF) of electrochemical H^+ gradients generated by H^+-ATPases at the plasma membrane (Brett et al., 2005; Ali et al., 2004; Padan et al., 2005; Hunte et al., 2005; Mager et al., 2011).

The PfMFS transporter of *P. funiculosum* has the closest relationship with MFS drug/H^+ antiporters (DHA) among identified MFS transporters. MFS drug/H^+ antiporters use the energy provided by proton motive force (PMF) of electrochemical H^+ gradients generated by H^+-ATPases at the plasma membrane to transport drugs from intracellular to extracellular and to couple the movement of H^+ from extracellular to intracellular (Paulsen et al., 1996). So far, several fungal MFS drug/H^+ antiporters were characterized and shown to mediate secretion of fungal natural toxins and fungicides. The secretion results in virulence of fungal toxins on plant and fungal resistance to toxins and fungicides (Alexander et al., 1999; Pitkin et al., 1996; Callahan et al., 1999; Hayashi et al., 2002; Prasad and Kapoor, 2005; Roohparvar et al., 2007; Liu et al., 2012). In contrast to drug/H^+ antiporters, some drug antiporters from the multidrug and toxic compound extrusion (MATE) family extrude toxic compounds using an electrochemical gradient of Na^+ established by a Na^+-ATPase and therefore acts as drug/Na^+ antiporters (Morita et al., 2000; Otsuka et al., 2005). In fungi, Na^+-ATPase, which was named ENA (exitus natra: exit of sodium), was found in almost all fungi and is P-type ATPase of the fungal-specific P2D subtype (Haro et al., 1991; Palmgren and Nissen, 2011). ENA ATPase localizes to the plasma membrane, where it uses ATP hydrolysis to extrude Na^+ from the cell, resulting in generation of electrochemical Na^+ gradients, which are able to drive ion transport across the cell membrane by secondary active transporters (Haro et al., 1991; Benito et al., 1997, 2002; Rodriguez-Navarro and Benito, 2010).

The PfMFS transporter of *P. funiculosum* shares the highest identity and similarity with EmrD, whose 3-D structure was solved (Yin

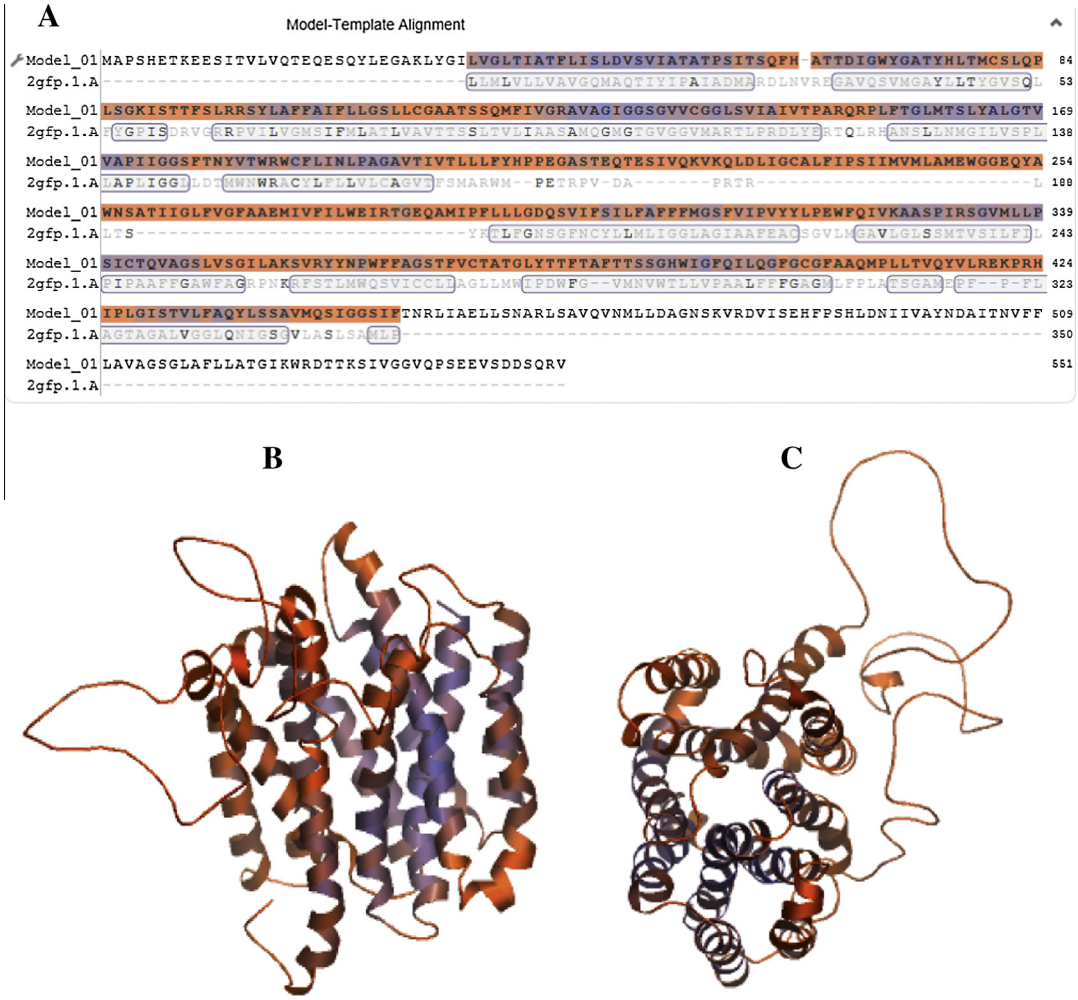

Fig. 7. Homology model of the PfMFS by Swiss-Model. (A) Structure-based sequence alignment and sequence conservation. Model_01: the PfMFS; 2gfp.1.A (PDB: 2GFP): *E. coli* EmrD. (B) The plausible 3-D model of the PfMFS was produced by using the 3-D structure of *E. coli* EmrD as a template (Yin et al., 2006). (C) View of the plausible 3-D model of the PfMFS looking towards the cytoplasm.

et al., 2006). EmrD is a close homolog to MDR MFS transporters, has 12 transmembrane α-helices, and is a H^+: drug antiporter from the DHA12 drug efflux subfamily within the MFS (Yin et al., 2006). The 12 transmembrane helices form a compact structure in the plane of the lipid bilaye. The four transmembrane helices face away from the interior (H3, H6, H9, and H12), and the remaining transmembrane helices form the internal cavity (Yin et al., 2006). The central cavity allowed substrates to bind in different orientations and locations. It was proposed that EmrD exposed a substrate through an intermediate occluded conformation between the open and closed forms of EmrD via the alternating access, "rocker-switch" mechanism (Yin et al., 2006). For a complete picture of the function of the PfMFS transporter of *P. funiculosum*, one needs to study crystal structure, proton transfer and protein conformational changes, which is challenging.

It is also interesting that what regulates gene expression of the PfMFS transporter of *P. funiculosum*. It is well-known that the biology of fungi is significantly influenced by environmental pH. The response to ambient pH includes the pH-conditional expression of several genes, which is directly or indirectly regulated by PacC, a transcription factor that regulates pH-conditional gene expression in fungi. PacC binds 5′-GCCARG-3′ sequences upstream of pH-responsive genes and either activates or represses transcription. Most data along with fungal genomes indicate that a pacC gene exists widely in fungi, containing *Penicillium* fungi (Denison, 2000; Penalva et al., 2008; Marcet-Houben et al., 2012; Zhang et al., 2013). However, *pacC* consensus 5′-GCCARG-3′ binding sites were not found in upstream of the PfMFS transporter of *P. funiculosum*. Whether the PfMFS transporter might be indirectly regulated by *pacC* or the binding specificity of *pacC* might be different is unclear.

The major facilitator superfamily (MFS) transporters are one of the most extensively studied families of transporters. Fungi dedicate a large amount of their genome to encoding the major facilitator superfamily transporters (Coleman and Mylonakis, 2009; Reddy et al., 2012). Recent genomic sequencing showed a number of MFS transporter genes in fungi. According to current publicly available annotations from some representative fungi, there are 356, 275, 301, 204, 251 and 335 MFS transporter genes in *Aspergillus nidulans*, *Aspergillus fumigatus*, *Stagonospora nodorum*, *Botrytis cinerea*, *Magnaporthe grisea* and *Fusarium gramainearium*, respectively. One of the most extreme examples is *Penicillium chrysogenum* the genome of which contains as many as 416 putative MFS transporter-encoding genes. These data indicate the phylogenetic diverse nature of MFS family transporters in fungi, which can be attributed to such factors as increased capacity to utilize a wide range of carbon and nitrogen sources, the capability for resistance to diseases and toxic compounds, and the ability to adapt to otherwise harsh environments, containing extreme low pH. We believe that new and more biological functions of the major facilitator superfamily transporters will be found in the future as research in biology progresses.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.06.002.

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